

## Transformation of Pneumococcal Types

### Introduction

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#### I. Brief historical review

- A. Griffith - 1928 - unencapsulated, avirulent R-variants of one specific type transformed into fully encapsulated and virulent cells of a different specific type.

Example and technique:

Type IIR → Type III S

R strain incapable of causing fatal bacteremia

Heated Type III Suspension contained no viable organisms.

Thus, R cells acquired capsular structure and serological specificity of Type III.

Slide I. - illustrates phenomenon  
(Insert)

#### B. Confirmation of phenomenon

1. Newfeld + Levinthal - 1928 } abroad.
2. Baurheim - 1932
3. Dawson - 1930 - in laboratory

#### C. Induction in vitro

1. Dawson and Sia - 1931. (intact and heated cells)

Grew R cells in media containing anti-R serum and heated S cells.

2. Alloway 1932 - (cell-free extracts; T.P. in soluble form)  
Barkfeld filtered.

Hence in test tube as in mice, showed transformation induced and selectively determined by type specificity of S cells used.

#### D. Virus - fibroma → myxoma

1. Berry and Dedrick - 1936

Living fibroma virus + heat-killed myxoma → rabbits induced myxomatosis

2. Berry 1937

Induction by suspensions of washed elementary bodies of myxoma

3. Confirmation by number of investigators.

Present study - more detailed analysis of phenomenon; attempt to determine chemical nature of T.P.  
Model: Type II → Type III.

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II Cultural Conditions - certain conditions requisite.

A. Broth - optimal for growth. Individual and unpredictable variations in capacity to support transformation. These largely eliminated by charcoal adsorption (MacLeod and Mirick)

B. Serum or serous fluid

1. Anti-R serum first used because of capacity to promote reversion of R → homologous S.
2. Alloway used ascitic and chest fluid and normal swine serum (contain anti-R)
3. Present study - human pleural and ascitic fluids used.  
Effectiveness of different lots varies. Differences not dependent on content of R antibodies, suggesting other factors involved.
4. Enzyme - various animal sera irrespective of immune properties contain enzyme that destroys T.P.  
Enzyme inactivated at 60-65°C. Heating may render ineffective sera effective.
5. Unknown factor - suggest by fact that some sera containing anti-R and enzyme heat inactivated still fail to support transformation. Nature of unknown factor undetermined.
6. Properties of serum - stable; may be stored in refrigerator many months and retain original effectiveness.

Recognition of serum factors and properties facilitated standardization of cultural conditions required for consistent and reproducible results.

C. R-strain (R36A) - derived from Ph. Type II

1. Characteristics - relatively fixed in R-phase; never spontaneously reverts. Repeated attempts to cause reversion unsuccessful.  
Strain susceptible to transformation to variety of different S types. (I, III, IV and XIV)
2. Dissociation - on serial transfer in blood broth R-strain undergoes spontaneous dissociation → number of variants distinguishable by colony form. Only R36A susceptible; other variants all inactive.  
Emphasizes care requisite in selection of suitable R-variant.

3. Intracellular enzyme. - pneumococcal cells release upon autolysis an enzyme that destroys activity of transforming extracts. Important in the cultural conditions of inducing transformation and in the extraction of T.P. from pnc. cells.

To obtain consistent reproducible results; bear in mind:

1. R-strain may undergo spontaneous dissociation giving rise to other variants incapable of responding to transforming stimulus.
2. Pnc. cells contain intracellular enzyme which when released destroys activity of T.P.

Hence, important to select reactive strain and to prevent destructive changes associated with autolysis.

#### D. Transforming principle.

Quantitative titration of activity of transforming material.

Sterile material serially diluted in saline at neutral pH. 0.2cc of each dilution added to three or more tubes containing 2cc of broth to which 10% serous fluid has been added. Tubes seeded with 0.05cc of a  $10^{-4}$  dilution of a 5-8 hour blood broth culture of the R-strain (R36A).

Cultures incubated  $37^{\circ}$  for 18-24 hours. Anti-R in serum medium causes the R-cells to agglutinate during growth. The agglutinated clumps settle to bottom of tube leaving clear supernatant. When transformation occurs, encapsulated S cells unaffected by the R antibodies grow diffusely, supernatant becoming uniformly turbid. By inspection alone can distinguish tentatively between positive and negative results.

All cultures plated on blood agar for further identification. Differentiation of colonies of parent R cells and those of transformed S organisms striking - latter large, glistening mucoid typical type III

Slide 2 - colonies

### III. Preparation of T.P.

- A. Source material { a 75 liter lot of Type III pneumococcus.  
culture of  
Young, actively growing (16 hr.)  
2. Collected on Sharples centrifuge.  
3. Cells resuspended in Saline - Heat Killed 65°C 30'.  
This Temperature inactivates Enzyme Known to destroy T.P.

### B. Extraction

1. Heated cells crushed 3x with Saline, removing  
large excess Capsular polysaccharide, much protein, ribonucleic acid  
and C polysaccharide - 10-15% loss of Transforming material -  
2. Extracted with saline containing 0.5% sodium  
deoxycholate, by mechanical shaking. Repeated 3x.  
3. Extracts combined, precipitated by excess ethyl  
alcohol. Ppt - floating fibrous mass. Redissolved in  
saline.

### C. Deproteinization and removal of S polysaccharide

1. Preliminary deproteinization by Sevag chloroform method. About 3X  
2. S<sub>III</sub> Enzyme hydrolyzes Type III capsular polysaccharide. Enzymatic  
breakdown usually complete 4-6 hours - evidenced by loss of serological  
activity.  
3. Reprecipitated by alcohol. Deproteinization repeated until no further  
protein-chloroform gel at interface.

### D. Alcohol fractionation

1. Dropwise addition of absolute ethyl alcohol with constant stirring  
2. At critical concentration - 0.8-1.0 volume - fibrous strands  
separate out and collect on rod.  
3. Repeated 4-5 X.  
4. Yield of fibrous material = 10-25 mgm per 75 liter lot and  
represents major portion of active material in crude extract.

### E. Effect of Temperature

1. Extraction less efficient but activity best preserved when  
procedures are carried out at 0°-4°C.

## IV. Analysis of Purified Material

### A. General properties

1. Saline solutions. (single) - colorless, clear in diffuse light. In strong transmitted light, silky sheen on stirring.
2. Preservation - saline solutions retain activity 2-4°C at least longer periods in frozen state in CO<sub>2</sub> cabinet. 3 months. In aqueous solution rapid decrease in activity; completely inert in few days.  
Material precipitated from saline solution by alcohol and stored under alcohol remain active for long periods.
3. Effect of temperature - withstands 20-60 min. 65°C. Higher temperatures not tested.
4. Effect of pH - activity rapidly lost at acidities greater than pH. 5. Best preserved at neutral or slightly alkaline reaction.

### B. Qualitative Chemical Tests

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1. Biuret and Millon tests negative - even on dried material
2. Dische diphenylamine reaction for desoxyribose strongly positive.
3. Orcinol test (Bial) for ribose weakly positive. However, in similar concentrations pure preparations of desoxyribonucleic acid of animal origin prepared by different methods give Bial reaction of corresponding intensity.
4. Lipids - no specific tests. Crude material repeatedly extracted with alcohol and ether -12°C without loss of activity. Repeated alcohol ppt<sup>n</sup> and treatment with chloroform results in no decrease in activity.

### C. Elementary Chemical Analysis (Dr. Elek)

#### Slide 3

N/p ratio varies from 1.58 - 1.75. Average value 1.67.

Close agreement with that calculated on the basis of the theoretical structure of sodium desoxyribonucleate.

N/p ratio indicate little protein or other substance containing N or P present, otherwise ratio would be considerably different.

### D. Enzymatic analysis

1. Crystalline enzymes (Northrop and Kunitz)

Trypsin, chymotrypsin and ribonuclease - no effect on activity.

Pepsin not tested because pH required for action itself inactivates.

## 2. Crude Enzymes

Dog intestinal mucosa - Levenson + Dillon - polynucleotidase prep<sup>b</sup>  
 Rabbit bone phosphatase - Martland + Robison  
 Swine kidney phosphatase - Albers, A. + E.  
 Pneumococcus autolytic  
 Normal dog + rabbit serum

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### Slide 4

(Dr. Mirsky - desoxyribonuclease)

Parallelism between enzyme that causes depolymerization of known samples of desoxyribonucleic acid and that which destroys activity of T.P. Irrespective of phosphatase or esterase activity, only those preparations shown to contain an enzyme capable of depolymerizing authentic samples of DRNA were found to inactivate T.P.

## 3. Differential Heat Inactivation of Dog. and Rabbit Serum

Greenstein + Jenrette show that sera of several different mammalian species contain enzyme which causes depolymerization of DRNA. Greenstein has termed this desoxyribonucleo-depolymerase. Action of enzyme measured by decrease of viscosity of mixtures of enzyme and nucleate in viscosimeters

### Slide 5

Dog + rabbit serum

Tested on partially purified preparation of transforming material.

Data show that both dog and rabbit serum unheated is capable of completely destroying transforming activity. However, when dog serum is heated at 60° or higher for 30 minutes there is no loss of transforming activity. That is, the serum enzyme responsible for destruction is completely inactivated at 60°C. In contrast exposure to 65°C is required for complete inactivation of the corresponding enzyme in normal rabbit serum.

Same samples of serum also tested for depolymerase activity on sodium desoxyribonucleate.

### Slide 6

Depolymerization followed by reduction in viscosity and progressive decrease in acid precipitability of the nucleate.

Data in Slide 6 show the differential heat inactivation of the depolymerase in dog and rabbit serum. With unheated serum of both species, viscosity fell to that of water in 5-7 hours.

1. Dog serum heated at 60 and 65°C - no significant reduction in viscosity after 22 hours.

On the other hand, heating rabbit serum at 60° merely reduced the rate of reaction. The depolymerase was completely destroyed at 65°

Thus, striking parallelism between temperature of inactivation of depolymerase and that of the enzyme which destroys T.P. (shown in slide 5) Difference in temp. of inactivation not a general property of all enzymes in these sera as evidenced by experiments on heat inactivation of esterase in these same samples. In latter instance, the results are the reverse of those observed with depolymerase, since the esterase of rabbit serum is almost completely destroyed at 60° while that of dog serum is only slightly affected by exposure to this temperature.

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#### 4. Inhibition by NaF

Of various substances tested only sodium fluoride found to exert significant inhibition known to destroy T.P. Irrespective of whether this enzyme derived from pneumococcal cells, dog intestinal mucosa or normal animal sera, its activity is inhibited by fluoride. Similarly, found that fluoride inhibits the enzyme causing depolymerization of D.R.N.A.

5. The fact that transforming activity is destroyed only by enzyme preparations known to contain depolymerase for D.R.N.A. and further fact that in both instances the enzymes concerned are inactivated at the same temperature and are inhibited by fluoride provide additional evidence for the belief that T.P. is a nucleic acid of the deoxy ribose type.

#### E. Serological analysis

1. Progressive loss of serological activity with chemical purification, without corresponding loss of activity.
2. Highly purified T.P. - only faint trace reactions with high titer Type II rabbit antiserum. Indicates elimination during purification of such serologically reactive substance as S, P, and C.
3. Contrast between biological activity and serological reaction.

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F. Physical chemical studies (Prep #44)

1. Ultracentrifugation (Dr. Rothen)

- a. Single, sharp boundary - indicating substance homogeneous and molecules uniform in size and very asymmetric.
- b. Biological activity sedimented at same rate as boundary
- c. Molecular weight - cannot be accurately determined pending measurement of diffusion constant and partial specific volume. However, Tennent and Vilbrandt have determined the diffusion constant of several preparations of T.N.A., the sedimentation rate of which closely agrees with that observed in the present study. Assuming that the asymmetry of the molecules is the same in both instances, it is estimated that the molecular weight is of the order of 500,000.

2. Electrophoresis (Dr. Sheldovsky)

- a. Single electrophoretic component of relatively high mobility comparable to that expected of a nucleic acid.
- b. Transforming activity associated with this fast-moving component giving boundary.
- c. Thus, in both the centrifugal and electrical fields, behavior of active material consistent with the concept that biological activity is a property of the highly polymerized nucleic acid.

3. Ultra-violet spectroscopy (Dr. Lavin)

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- a. Absorption curves showed maxima at  $2600\text{\AA}$  and minima in the region of  $2350\text{\AA}$
- b. Findings characteristic of a nucleic acid.

G. Quantitative determination of biological activity

1. Various prep's of highly purified material active in amounts ranging from 0.02 - 0.003 micrograms

2. Slide 7. Titration of activity of prep? #44 - prepared in cold; high activity; N/p ratio = 1.58.

On the basis of dry weight - 0.003 micrograms is effective in transformation. Since the reaction system containing the 0.003 micrograms has a volume 2.25cc this represents a final concentration of the purified substance of 1 part in 600,000,000